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An Acid Catalyzed Reversible Ring-Opening/ Ring-Closure Reaction Involving a Cyano-Rhodamine Spirolactam

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An acid catalyzed reversible ring-opening/ring-closure reaction involving a cyano-rhodamine spirolactam†

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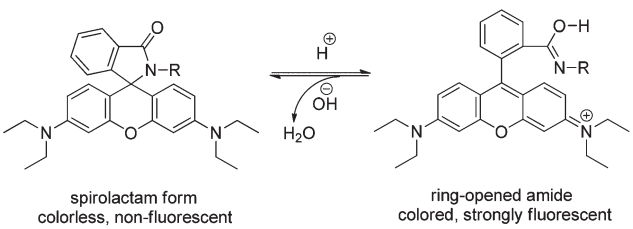
Cyanamide was introduced into the rhodamine spirolactam framework to produce a colorless and non-fluorescent compound RBCN. It shows a reversible ring-opening/ring-closure process in response to the solution pH, which exhibits an "ON/OFF" switching in its fluorescence. Different from other rhodamine-type dyes, the ring-open form of RBCN is stable in protic solvents under neutral, near neutral and basic conditions, showing a pink color and very strong fluorescence. We also demonstrated the potential of RBCN in live cell imaging.

Chromogenic and fluorogenic signaling sensors for the detection and measurement of target analytes have been widely investigated in many areas such as biochemistry,[1a,b] medical diagnosis,[1c] and environmental analysis.[1d] The chromogenic signaling module can easily offer naked eye detection by color changes while the fluorescent methods can give high sensitivity and shorter response time, and thus can offer a real-time monitoring of a process occurring at different time scales. Hence, sensors of this kind have attracted enormous interest in the past decade.[1a,e]

The rhodamine based dyes have recently been extensively used for molecular recognition and molecular devices[2a] due to their excellent photophysical properties, such as high extinction coefficients, excellent quantum yields, great photostability, and relatively long emission wavelengths. The well-known rhodamine spirolactam framework is an ideal system to construct OFF–ON sensors because the ring closed spirolactam form is non-fluorescent and colorless while the corresponding ring open form is strongly fluorescent and pink colored.[2] It is convenient to introduce the demanded recognition site into the spirolactam system and develop it as the dual channel (chromogenic and fluorogenic) OFF–ON sensor for detection of various analytes such as transition and heavy metal ions,[3] anions,[4] amino acids[5] and reactive oxygen species.[6]

The commonly existing form of the rhodamine spirolactam derivative is colorless and non-fluorescent in common (protic or aprotic) solvents, which structurally equilibrates to a colored and highly fluorescent ring-opened amide form upon analyte-induced binding or reaction.[2] For example, an acidic pH can usually activate a non-fluorescent rhodamine spirolactam to be a highly fluorescent rhodamine derivative (Scheme 1). In the literature, this kind of spirolactam ring was usually formed by condensation with an aliphatic or aromatic amine, hydrazine or hydrazone that linked with a variety of receptors.[2] In our study, a strong electron withdrawing group, the cyano group which has quite different electronic properties compared with that in the literature, was introduced into the spirolactam framework. Since the strong electron withdrawing effect of the cyano group can stabilize the cyanamide anion both inductively and by resonance delocalization (Scheme 2),[7]



Scheme 1 The reversible process of the rhodamine derivative controlled by pH. The acidic condition can result in the transition from spirolactam to ring-opened amide.

Scheme 2 Resonance delocalization of the cyanamide anion.

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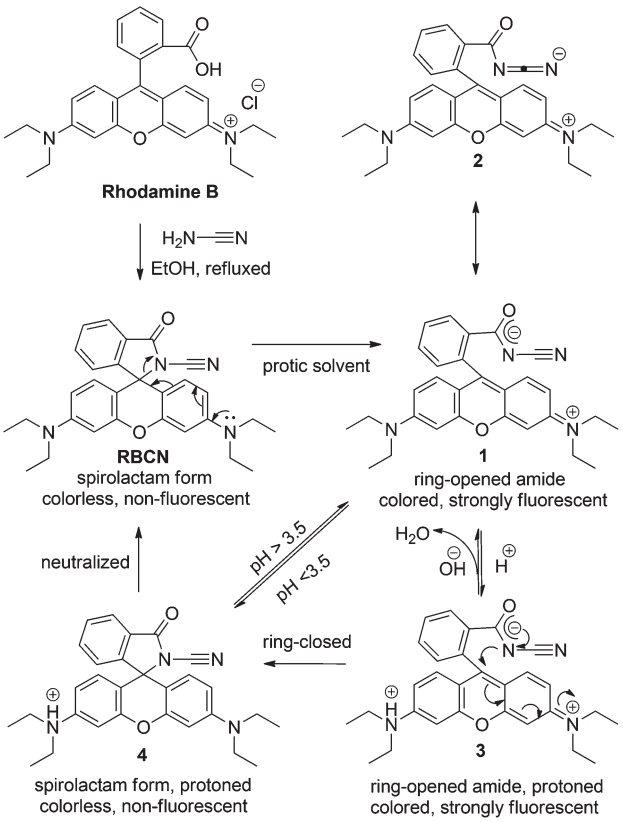
the introduction of cyanamide would thus result in very different fluorogenic properties for **RBCN**.

The compound **RBCN** was easily synthesized by condensation of the commercially available rhodamine B and cyanamide in refluxed ethanol solution (Scheme 3) with a moderate yield of 55%. Its structure was confirmed by ^{1}H-NMR, ^{13}C-NMR, and MS analysis. The X-ray crystal structure[8] (see Table S1 and Fig. S1† for details) clearly represents the unique spirolactam ring structure (Fig. 1), which is also supported by the characteristic peak near 69.3 ppm (spiro-carbon) in the ^{13}C-NMR spectra of **RBCN** in $CDCl_3$. The absorption spectrum of **RBCN** in different solvents (Fig. S2†) shows that there are evident absorption peaks in the protic solvents (pink color, Fig. S3†), such as MeOH (peak at 548 nm), EtOH (peak at 547 nm) and H_2O (peak at 558 nm), but no evident absorption peaks in the aprotic solvents. As shown in Fig. 2, similar results can also be observed from the fluorescence spectra. **RBCN** in protic solvents displays a strong emission (orange fluorescence) at 572 nm in MeOH, 569 nm in EtOH and 583 nm in H_2O and no evident emission peaks were observed in aprotic solvents. Both the absorption and emission properties confirm that **RBCN** exists as a ring-open amide in these protic solvents (for **RBCN** in other protic solvents, see photos in Fig. S3†).



Scheme 3 Synthesis of **RBCN** and the proposed mechanism for the acid catalyzed ring-opening/ring-closure process.

As references reported, all the rhodamine spirolactam derivatives are colorless and non-fluorescent in common (protic or aprotic) solvents and then will act as chromogenic and fluorogenic sensors in the target analytes sensing process.[2] Only Bag's group developed a FRET signaling system based on rhodamine B and nitrobenzofurazan fluorophores with an acyclic amino receptor,[9] that will display a prominent enhancement in absorption and emission induced by H_2O, MeOH or EtOH. They pointed out that the H-bonding interactions[10] of these protic solvents with the acyclic amino receptor might play a vital role in the ring-open process. In the case of **RBCN**, three points should be noticed: (a) the strong polarity of these solvents is favorable for the intramolecular charge separation in its ring-open state, (b) the ring-open amide was stabilized by the strong electron withdrawing cyano group, and (c) potential H-bonding interactions of the protic solvents with the cyanamide moiety[7d] may also contribute to stability of the ring-open state.

Although there was very weak fluorescence for **RBCN** in aprotic solvents such as acetone, CH_3CN, THF, DMF and DMSO, the trace amounts of water can lead to an evident fluorescence enhancement (Fig. S4†). This can be even detected by the naked eye under UV-light (Fig. S4b† inset). It is well known that rhodamine spirolactam derivatives can act as fluorescent sensors for pH sensing under acidic conditions due to the ring-open process induced by the proton (Scheme 1).[11]

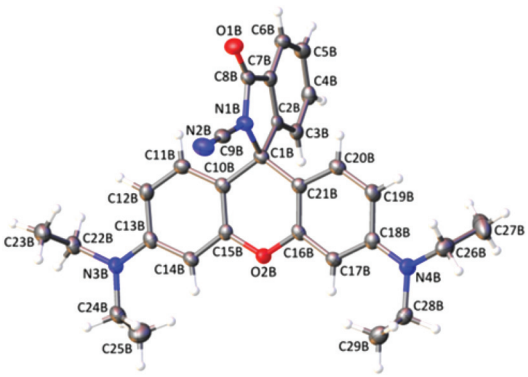


Fig. 1 X-ray crystal structure of **RBCN**. Displacement ellipsoids are shown at the 50% probability level.

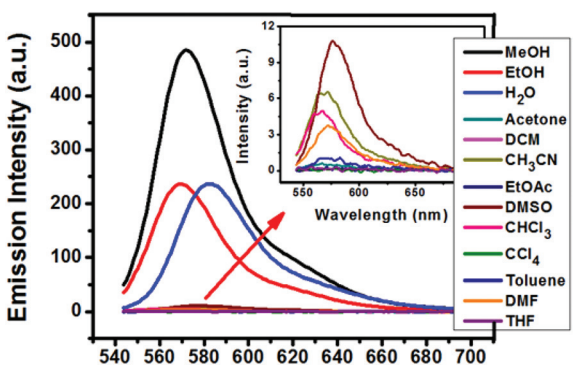


Fig. 2 Fluorescence spectrum of **RBCN** (10 μM) in different solvents. λ_{ex} = 530 nm.

However, in our **RBCN** system, the cyano group makes it display very different spectroscopic properties under acidic conditions as compared with similar rhodamine spirolactam compounds. **RBCN** was strongly fluorescent in the basic, neutral and near-neutral range as shown in a pH titration experiment (Fig. 3c). When the pH was decreased below 3.5, the fluorescence decreased and was completely quenched at pH ~ 1.2, accompanied by similar changes of absorption intensity. Fig. 3 displays the absorption (Fig. 3a) and fluorescence (Fig. 3b) spectrum changes of **RBCN** from pH 1.2 to 4.2. These results show an inversed pattern of pH-dependent changes in fluorescence and absorption changes compared with the traditional rhodamine spirolactam analogs.

Recently, more efforts have been made on the development of "acidic" probes with high sensitivity, good photostability and excellent membrane permeability based on the rhodamine spirolactam framework.[11] However, all of them display fluorescence decreases when pH was adjusted from low to high in their working range (commonly around pH 4.0–6.0). Herein, we developed **RBCN** as a sensitive fluorescence 'turn-on' sensor for detection of pH changes under more acidic conditions. As shown in Fig. 3c, the fluorescence signals increased when pH was adjusted from 1.2 to 4.0 and they were linearly correlated (R^2 = 0.994) to pH changes from 2.0 to 3.1 (Fig. 3c inset).

Additionally, the pH-dependent change in the fluorescence and absorption of **RBCN** is reversible and can be repeated for multiple cycles (Fig. 4). We also used NMR spectra to monitor the ring-opening/ring-closure process involving **RBCN** (Fig. S5 and S6†). The characteristic peak at 69.3 ppm in ^{13}C-NMR is ascribed to the signal of the spiro-carbon atom which demonstrated the ring-closed form of **RBCN** in $CDCl_3$. When CD_3OD was added into this sample, the peak for the spiro-carbon atom disappeared and shifted to 143.7 ppm, with the color changing from light pink to red which indicated the ring-open form of **RBCN**. After adding 16.7% (v/v) of DCl[12] and mixing well, the light pink color of the **RBCN** sample was immediately recovered. Moreover, the peak at 143.7 ppm shifted back to the alkyl region on the ^{13}C-NMR spectrum around 67.2 ppm. The recovery of spiro-carbon is a strong support for the ring-closure of **RBCN**.

We then proposed an acid catalyzed reversible ring-opening/ring-closure process for **RBCN** in a protic solvent. As shown in Scheme 3, the resonance structures (**1** and **2**) and the H-bonding interaction from the protic solvent can stabilize the ring-open state of **RBCN** even under basic conditions. When pH was decreased to lower than 3.5, the H-bonding interaction was effectively quenched by the increasing of H^+ and the *N*-cyano amide moiety was free. It displayed lower basicity but better nucleophilicity[7] (compared with amides in other similar

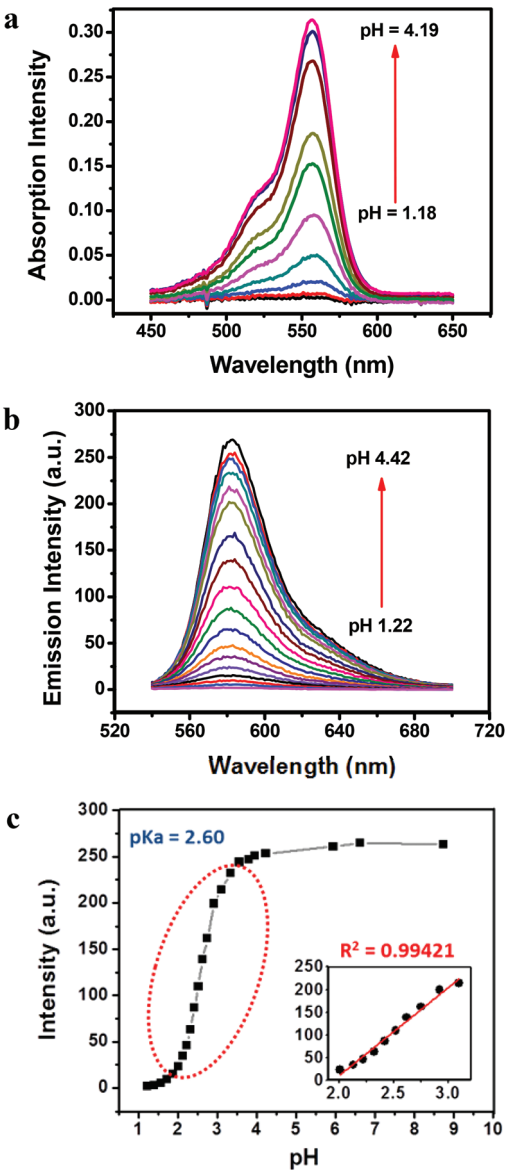


Fig. 3 The absorption (a), fluorescence (b) spectral changes and fluorescence intensity (at 583 nm) changes (c) of **RBCN** under different pH conditions. The inset of (c) shows the linear relationship between fluorescence intensity and pH from 2.0 to 3.1. Conditions: **RBCN** (10 μM) in water with 0.2% acetonitrile. pH was adjusted by 0.1 M and 2.0 M $HCl_{(aq)}$ and $NaOH_{(aq)}$, λ_{ex} = 530 nm, λ_{em} = 583 nm.

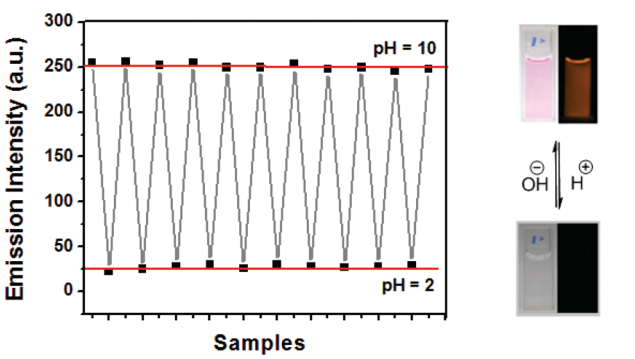


Fig. 4 The reversible pH-dependent fluorescence changes (left) and photos (right) of **RBCN**. Conditions: 10 μM of **RBCN** in water with 0.2% acetonitrile at pH = 2 and 10. The pH was adjusted by $HCl_{(aq)}$ and $NaOH_{(aq)}$. λ_{ex} = 530 nm, λ_{em} = 583 nm.

rhodamine spirolactam compounds) that is favorable for the ring-closure reaction. Additionally, the diethylamino group was possibly protonated to form **3** and the electron-deficient nature of the xanthene moiety was also favorable for the nucleophilic attraction of the cyanamide moiety, thus inducing the ring-closed reaction to form the protonated **4**, which can be neutralized to give the initial **RBCN**. Since the protonation of **3** and **4** can be easily removed by adjustment of pH to higher than 3.5 and generates the ring-open form **1**, the acid catalyzed ring-closure process of **RBCN** is reversible. The peak for methylene carbon and peaks for protons on the xanthene framework (Fig. S5c and S6c†) all displayed evident downfield shifts that supported the protonation of ring-closed **RBCN**.

Finally, we applied **RBCN** to the SW620 cell (human colon cancer cell line) to examine whether it can work for cell staining. SW620 cells were washed with PBS buffer and costained with **RBCN** and LysoTracker Green (LysoTracker Green DND-26) or MitoTracker Green (MitoTracker Green FM) from Invitrogen. The cells imaging was taken by confocal fluorescence microscopy to probe the distribution of **RBCN** relative to LysoTracker Green (Fig. 5 top) or MitoTracker Green (Fig. 5 bottom) in live cells. The co-staining experiments revealed that **RBCN** mostly stained the lysosomes, exhibiting staining patterns that were nearly identical to that of LysoTracker Green (Fig. 5 top). The colocalization of **RBCN** with LysoTracker Green, instead of MitoTracker Green, suggested that **RBCN** displayed a better selectivity for lysosomes compared with mitochondria in live cells, which is consistent with literature reports that rhodamine spirolactam based sensors prefer lysosome in live cells.[11b,c]

In summary, we presented the synthesis of **RBCN**, a new rhodamine spirolactam derivative with a cyano group. The strong electron withdrawing group makes **RBCN** display much different spectral properties as compared with other reported similar rhodamine spirolactams. In protic solvents, **RBCN** exists as a ring-open state under neutral, near neutral and basic conditions. From the pH titration experiment, **RBCN** shows an acid catalyzed reversible ring-opening/ring-closure process in aqueous solution. To the best of our knowledge, this is the first report about the acid catalyzed ring-opening/ring-closure involving rhodamine spirolactam derivatives. Finally, we also demonstrated that **RBCN** displayed a better selectivity for lysosomes compared with mitochondria in live cells.

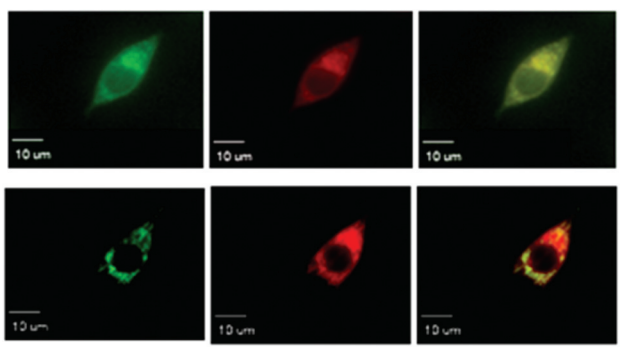


Fig. 5 Intracellular distribution of **RBCN** as compared to LysoTracker Green or MitoTracker Green. SW620 cells were co-stained with LysoTracker Green and **RBCN** (top) or MitoTracker Green and **RBCN** (bottom). Green fluorescence shows the localization of LysoTracker Green or MitoTracker Green while red fluorescence indicates the localization pattern of **RBCN**. Merging of the fluorescence of LysoTracker Green or MitoTracker Green and **RBCN** was shown in yellow. Concentration: 5 μM for **RBCN**, 15 nM for LysoTracker Green and 500 nM for MitoTracker Green, respectively. Scale bars are 10 μm.

Acknowledgements

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